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Head and thoracic transformations caused by ectopic expression of *Antennapedia* during *Drosophila* development

GREG GIBSON and WALTER J. GEHRING

Department of Cell Biology, Biozentrum, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Summary

Segmental identity in *Drosophila* is controlled by the activities of the homeotic genes. One such gene is *Antennapedia*, which is required for the proper development of the thoracic segments. Alteration of *Antennapedia* expression either in mutants, or artificially using an inducible promoter, can lead to alterations of segmental identity. In this report, we present the consequences of ectopic expression of the *Antennapedia* gene under the control of a heat-shock promoter, at distinct stages throughout *Drosophila* development. In young embryos, up to the stage of germband retraction, the ubiquitous expression of the Antennapedia protein causes a range of effects throughout the embryo, including failure of head involution, induction of extra denticles on the dorsal surface of the head and disruption of the prothoracic denticle belts. In older embryos, it results in larval lethality. Heat shocks during larval development can lead to defects in leg formation, but no alterations in leg identity have been observed. However, clear transformations of head towards second (meso-) thoracic segment can be induced in early third instar larvae. There is a distal-to-proximal temporal response to ectopic *Antennapedia* expression in the antennal disc, as evidenced by successive transformations of the arista, third antennal segment, second antennal segment and occiput towards their corresponding leg and dorsal thoracic structures. Overproduction of Antennapedia protein during the pupal stage is generally lethal. Comparison of the homeotic transformations in, and Western analysis of, different lines suggests that a relatively large amount of Antennapedia protein is required to cause antenna-to-leg transformations, and further argues that, in general, developmental programmes in the insect are well buffered against the effects of ectopic homeotic gene expression. Immunodetection of Scr and Antp protein also allows us to interpret the results in light of the hypothesis that the various selector genes compete with one another to control not only their own expression, but also that of downstream genes. The role of *Antennapedia* in imaginal disc determination is also discussed.

Abbreviations: *Antp*, *Antennapedia* gene; Antp, Antennapedia protein; *Scr*, *Sex combs reduced* gene; *Ubx*, *Ultrabithorax* gene; ANT-C, Antennapedia gene complex; BX-C, bithorax gene complex.

Key words: Antennapedia, *Drosophila* development, ectopic expression, segmental identity, thoracic transformation.

Introduction

Genetic studies of *Drosophila melanogaster* have identified a group of genes which appear to control the identities and sequence of the body segments, but little is known about how they work at a molecular level. The homeotic selector genes are defined by mutations that lead to the 'homeotic' transformation of one segment towards another and reside in two complex gene clusters, the Bithorax (BX-C) and Antennapedia (ANT-C) complexes (E. Lewis, 1978; Kaufman *et al.* 1980; R. Lewis *et al.* 1980*a*,*b*). Genes of the BX-C control the identities of segments in the abdomen and posterior thorax, while those of the ANT-C are involved in the determination of the identities of anterior thoracic and head structures. Lewis (1978) has proposed a model that suggests that in each segment of the developing organism, a different combination of these genes are activated and Garcia-Bellido (1975) has argued further that they act by controlling, or 'selecting', the expression of other groups of genes which actually 'realize' the

identities of the segments. Both ideas are generally supported by molecular data which show that the homeotic selector genes share the homeobox and are indeed expressed in specific regions of developing embryos, and suggests that they encode DNA-binding proteins which can regulate gene expression (for review, see Gehring & Hiromi, 1986). Based upon DNA sequence comparisons between the homeobox and gene regulatory proteins in yeast and prokaryotes, the competition hypothesis has been put forward (Shepherd *et al.* 1984; Laughon & Scott, 1984; Gehring, 1987), which further proposes that segmental identity is in part determined by competition, between the various homeotic selector genes, to bind to the same promoters and to regulate downstream gene expression. However, just which genes they regulate, and how they do so, remains to be elucidated.

The classical approach to examining the functions of the homeotic selector genes has been to ask what happens when the domains of expression of the homeotic gene products are permanently altered. These studies have revealed the general principle that loss-of-function mutations in *Antp* and the BX-C cause transformations toward anterior identity, while gain-of-function mutations cause posterior transformations (Lewis, 1978). This principle can to a large extent be explained by the genetic (Struhl, 1982, 1983) and molecular (Hafen *et al.* 1984; Struhl & White, 1985) evidence that the homeotic genes interact to control one another's expression: more posterior genes are thought in general to repress more anterior genes, at least in the posterior body segments. The *Antennapedia* (*Antp*) gene, for example, is known to be required for the proper development of the thoracic segments, where it seems also to repress the activity of head-determining genes (Denell *et al.* 1981). Loss-of-function alleles of *Antp*, when homozygous or hemizygous, show transformations of meso- and meta-thorax towards prothorax (and even posterior head: Schneuwly & Gehring, 1985), possibly as a result of derepression of the *Scr* gene in those segments in the absence of the *Antp* gene product (Struhl, 1982). In addition, the dominant *Antp* alleles produce a range of homeotic transformations of anterior regions, most notably of antenna towards mesothoracic leg (Hazelrigg & Kaufman, 1983). These mutant phenotypes have been shown to be caused by inappropriate expression of *Antp* in the eye–antennal disc (Jorgensen & Garber, 1987). In the case of the $Antp^{73b}$ allele, this results from the fusion of *Antp* coding sequences with the promoter of another gene which is normally expressed in eye–antennal imaginal discs in third instar larvae and pupae (Frischer *et al.* 1986; Schneuwly *et al.* 1987*b*). Similarly, ectopic expression of the *Ubx* gene in the posterior mesothorax of *Cbx* mutants causes a transformation of wing to haltere (White & Akam, 1985). Alteration of the pattern of expression of a homeotic selector gene is, then, sufficient to cause a change in segmental identity.

Questions remain as to precisely when, and for how long, during development the incorrect expression is required for a homeotic transformation to occur; which tissues are susceptible to the overexpression of a gene such as *Antp*; and how much gene product (presumably protein) is required? We sought to address these questions through transient expression of the *Antp* gene in all cells of the organism at precise stages throughout *Drosophila* development. As reported by Schneuwly *et al.* (1987*a*), when the *Antp* cDNA is placed under the control of a heat-shock promoter, it is possible to induce a homeotic transformation of antenna to leg by giving heat shocks to early third instar larvae.

Here we extend that study to show that there is a temporal sequence of response in the antenna to Antp protein, resulting in distal-to-proximal transformations towards (meso-) thoracic structures. Further, it is concluded that overproduction of Antp protein has no effect on abdominal development. A detailed analysis of the effects of incorrect *Antp* expression during early embryogenesis again shows that only structures anterior to the normal domain of *Antp* expression are affected, and a strong transformation of prothorax towards mesothorax is observed. The results also define a biological assay for the function of the Antp protein, which will form the basis for further studies on the molecular mechanisms by which homeotic genes control segmental identity.

Materials and methods

Fly stocks

Flies were raised at 25°C on standard medium containing cornmeal, sugar, yeast and agar. The heat-shock–*Antp* strains used have been reported previously (Schneuwly *et al.* 1987*a*), but are also briefly described here. For a control, we used a strain (HTC-1) containing an insert of the pHT4 vector, which consists of the heat-shock promoter and trailer from the *Drosophila hsp70* gene, separated by a *Kpn*I site, inserted into the Carnegie 20 vector of Rubin & Spradling (1982). The heat-shock–*Antp* strains, H4, H22, H36 (maintained as a balanced stock over CyO) and H45 carry stable integrations of the vector pHTA, which consists of the *Eco*RI fragment of *Antp* cDNA 909C (Schneuwly *et al.* 1986*a*) inserted into the *Kpn*I site of pHT4. As a further control, we used a strain (K104) carrying a deletion of the *Antp* gene from the *Kpn*I site in the homeobox to the *Eco*RI site at the 3′ end of the cDNA 909C inserted in the pHTA vector (A. Schier, G.G. and W.G., unpublished data), which produces a truncated Antp protein.

Embryonic heat shocks

Embryos were collected on yeasted apple juice plates for 1 h and allowed to mature for a further 2 h at 25 °C before dechorionation for 1 min in 3 % sodium hypochlorite. They were then submerged in water for staging under a binocular microscope at room temperature over the next half hour. Embryos were transferred in groups of 20–30 to fresh apple juice plates at the cellular blastoderm stage, when elongated nuclei were clearly visible at the periphery of the embryo, but before ventral furrow formation. This time was taken as 2½ h of development and all embryos were estimated to be within a 30 min period of development. They were then allowed to mature further at 25 °C until heat treatment. Heat shocks were delivered by transferring the embryos with a paintbrush to a drop of water on a cover slip, which was floated on a 37 °C water bath for the desired period of time. The embryos were finally allowed to mature completely at 25 °C on apple juice plates.

Larval heat shocks

Embryos were collected for 1 h on yeasted apple juice plates and allowed to mature at 25 °C. They were transferred within 1 h of hatching (taken as 22 h of development), in batches of 10, to yeasted cornmeal tubes and grown further at 25 °C. Unless otherwise stated, each batch of animals were given a single heat shock, simply by placing the tubes in a 37 °C incubator for the indicated period of time. 18 treatments at 4 h intervals from 24 to 92 h of development were spaced in the following manner: six collections, each divided into three batches, were made at 12 h intervals, and then one tube from each collection was treated 24, 28 or 32 h after the final collection. Similarly, for treatments from 96 to 168 h of development, the tubes were treated 96, 100 or 104 h after the final collection. Although this method does not stage the larvae precisely, it is rapid, and for each treatment, the effects on individual flies were generally found to be similar.

Analysis of structures

To examine cuticular phenotypes, embryos were, if necessary, carefully dissected out of their vitelline membranes using sharp tungsten needles and mounted in Hoyer's medium (van der Meer, 1977). They were lightly squashed to flatten the denticle belts, placed for 1 week at 55 °C and analysed using phase-contrast, dark-field or Nomarski optics. Antennae, legs or head parts were simply mounted in Faure's medium.

Western blots

Ten late third instar larvae were placed on a moist piece of filter paper in a Petri dish, which was placed in a 37 °C incubator for 1 h and allowed to recover for the indicated times at 25 °C. Eight of these larvae were then homogenized in a small glass/teflon homogenizer in 0·4 ml × 1 SDS lysis buffer (Laemmli, 1970), 0·2 ml of which was then boiled for 2 min, and stored at −20 °C. Samples were run on a 10 % SDS–acrylamide denaturing gel (Laemmli, 1970) and transferred to nitrocellulose according to Towbin *et al.* (1979). Hybridization to a rabbit anti-Antp antiserum (B. Dalle Carbonare, unpublished data) was followed by ^{125}I-protein A detection as described by Johnson *et al.* (1984).

Immunolocalization

Immunofluorescence was performed in a scaled-down version of the procedure described by Mlodzik & Gehring (1987), except that a mouse anti-Antp antiserum (obtained as described in Wirz *et al.* 1986) diluted 1:100 was used. The peroxidase staining (MacDonald & Struhl, 1986), using a rabbit anti-Scr antiserum (P. LeMotte, unpublished data) followed by peroxidase-coupled swine anti-rabbit antibody (DAKO-immunoglobulins), was performed in PBS containing 0·5 mg ml^{-1} diamino benzidine and 0·003 % hydrogen peroxide, and embryos were dehydrated through graded ethanol before mounting in methyl salicylate. Embryos were staged as described for heat shocks.

Results

It has previously been noted that ectopic expression of the Antennapedia protein under heat-shock control in early embryos causes a failure of head involution (Schneuwly *et al.* 1987*a*). In order to determine more precisely which structures are affected by ectopic expression of the protein, we have examined the cuticular phenotypes of embryos, from various stocks transformed with the heat-shock–*Antennapedia* constructs, subjected to varying degrees of heat shock at staged periods throughout embryonic development. As a control we used a strain, HTC-1, containing an insert of the heat-shock vector pHTA (Schneuwly *et al.* 1987*a*), which does not produce any exogenous protein. Unless otherwise noted, the heat treatments used in these experiments had no effects on development in this strain. The major effects in heat-shock–*Antp* embryos can be divided into three classes: inhibition of head involution, effects on dorsal head structures and effects on the thoracic denticle belts. These are described below.

(A) Antennapedia *causes failure of head involution in early embryos*

The most striking effect of overexpression of *Antp* in the first 9 h of development is that it causes a failure of head involution. To ease interpretation of the results, we will first give a general description of the formation of the head in *Drosophila* larvae: for a detailed description, see Turner & Mahowald (1979). Although the embryonic head shows signs of segmentation (in the form of three gnathal lobes) at the time at which external segmentation first becomes visible (around 7 h of development, at the so-called extended germ-band stage), by the time of hatching, a complex series of morphogenetic movements have taken the majority of head structures into the thorax. This process of 'head involution' results in a larva with an acephalic appearance. Nevertheless, it remains possible to identify a number of cuticular structures that belong to the head and which, in this study, have

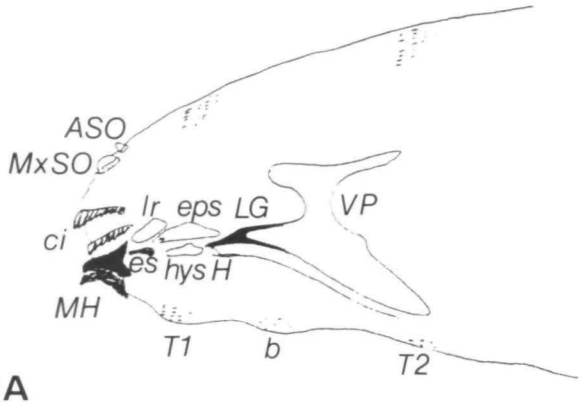
served as convenient markers for the extent of head involution. All of the terms used in the discussion below are those adopted by Jürgens *et al.* (1986) in their elegant study of the segmental organization of the larval head in *Drosophila*.

Perhaps the most prominent structure of the larval head is the cephalopharyngeal skeleton (CPS), a large, darkly pigmented structure adjacent to the pharynx and inside the first thoracic segment, within which various parts can be identified, two of which concern us here: the conspicuous paired vertical plates (*VP*) and the paired arms (the lateralgräten, *LG*) which link the remainder of the CPS to the morphological mouth (see generally Fig. 1A). A series of cuticular structures lines the LG anteriorly, including the paired hypostomal (*hys*) and ectostomal (*es*) sclerites ventrally, the unpaired median tooth (or labrum, *lr*) and epistomal sclerite (*eps*) dorsally, and a crossbridge, the darkly pigmented H-piece (*H*). At the orifice of the mouth itself are the two curved, tridentate mouth hooks (*MH*), and a series of non-pigmented sensory organs, including the two rows of cirri (*ci*), which extend dorsolaterally on the exterior of the larva towards the maxillary (*MxSO*) and antennal (*ASO*) sense organs (see Hertwick, 1931).

We have used Nomarski and phase-contrast optics to score for the presence of, and examine the location of, all of the above cuticular structures after heat shocks throughout embryogenesis. Depending on the precise time and extent of treatments, varying degrees of failure of head involution could be induced. While no structures were observed to fail to form completely, most (particularly the CPS and dorsal sclerites) were distinctly malformed, and in some cases difficult to identify unambiguously. In addition, as will be described below, a few new structures were induced, including extra denticle rows and hardened plaques. The consequences of failure of head involution are shown in Fig. 1B,H, which are schematic drawings of lateral and ventral views respectively of a head, illustrating all of the effects observed after 20 min heat shocks. Most strikingly, the CPS appears anterior to the thorax, with the ventral plates reduced to a swirl of hardened material and the lateralgräten now lying anteroventrally around the circumference of the head. The epistomal sclerite and labrum lie on the dorsal surface of the head, while the mouth hooks, cirri and antennomaxillary complex lie laterally.

For heat shocks of 5, 10, 15 or 20 min at 37°C at each stage of development in the strain H4 (which produces the strongest transformations), the resulting cuticular defects were highly reproducible from experiment to experiment and all embryos showed some defects. Phase-contrast photographs of the heads of representative mature embryos heat shocked after 3, 5, 7 or 9 h (for 20 min each) of embryogenesis are shown in Fig. 1C–F, respectively, and a wild-type head is shown for comparison in Fig. 1G. A comparison of the positions of structures such as the lateralgräten and the mouthhooks suggests that the failure of head involution was most severe if the heat shock was administered at 7 h of development (corresponding to germ-band extension: see Campos-Ortega & Hartenstein, 1985). At this stage, the lateralgräten failed to join, indicating that they had completely failed to move ventrally and anteriorly prior to involution (Fig. 1E). Similarly, the maxillary sense organs and mouth hooks were displaced more laterally than at other stages. The CPS formed an amorphous swirl of pigmented material at the anterior tip of the embryos, whereas if the heat shock was administered at earlier stages of development, distinct structures such as the ventral arms could be seen; in favourable preparations they extended towards the thoracic segments, indicating at least partial head involution (Fig. 1C). Clearly, a heat shock after 9 h of development caused only minor disruptions of head formation (Fig. 1F); even a brief heat shock during gastrulation (3 h of development) caused more severe disruptions, although at this stage the pharyngeal wall could always be seen to extend into the thorax (data not shown).

Fig. 1. Failure of head involution caused by heat-shock induction of *Antp* at different stages of embryogenesis. ×75. (A) Drawing of the head of a first instar larva, showing the normal locations of all of the structures scored in this study. *ASO*, antennal sense organ; *ci*, cirri; CPS, cephalo-pharyngeal skeleton; *eps*, epistomal sclerite; *es*, ectostomal sclerite; *H*, H-piece; *hys*, hypostomal sclerite; *LG*, lateralgräten; *lr*, labrum (median tooth); *MH*, mouth hooks; *MxSO*, maxillary sense organ; *VP*, vertical plates *A2*, 2nd abdominal segment; *T1*, prothorax (*b*, prothoracic beard); *T2*, mesothorax. (Adapted from Jürgens *et al.* 1986, fig. 3.) (B) Schematic drawing of a lateral view of a larva showing the locations of the various cuticular structures induced by heat shocks during the first 7 h of embryogenesis. Additional abbreviations: *ADB*, anterior denticle belt; *PDB*, posterior denticle belt, including dorsal- (*d*) and ventral- (*v*) type denticles. (C) Ventral view of the noninvoluted head of an H4 larva that had been heat shocked at 3 h of development for 20 min, photographed using phase-contrast optics. (D–F) Similarly show H4 larvae given a 20 min heat shock at 5, 7 and 9 h of development, respectively. Notice that head involution is most inhibited in E, since the lateralgräten *LG* fail to join and the sensory organs (*MxSO* and *ASO*) lie on the dorsal surface of the head, out of focus. In F, head involution is more advanced; the two lines extending into the prothorax represent the pharyngeal wall. (G) Wild-type head. (H) Schematic drawing of a ventral view of a larva as in B.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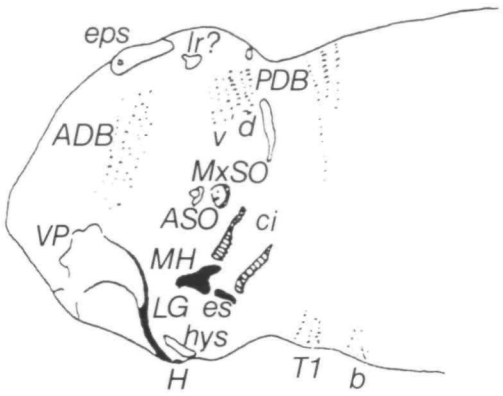
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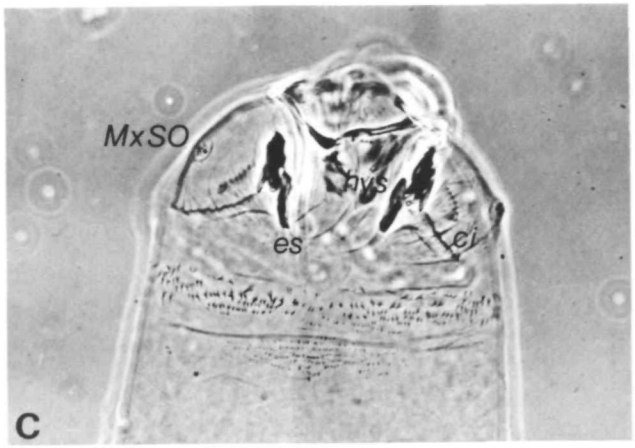
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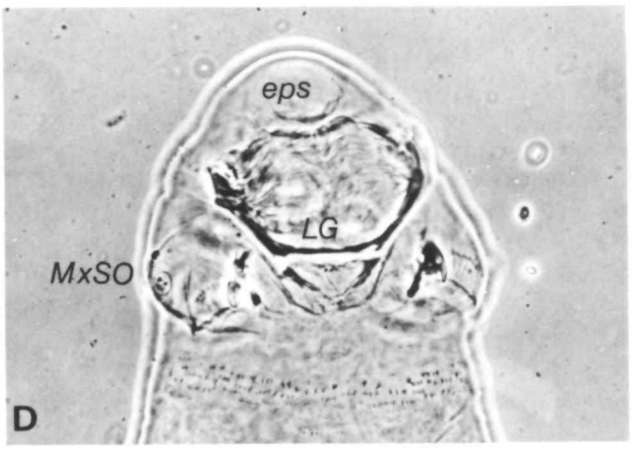
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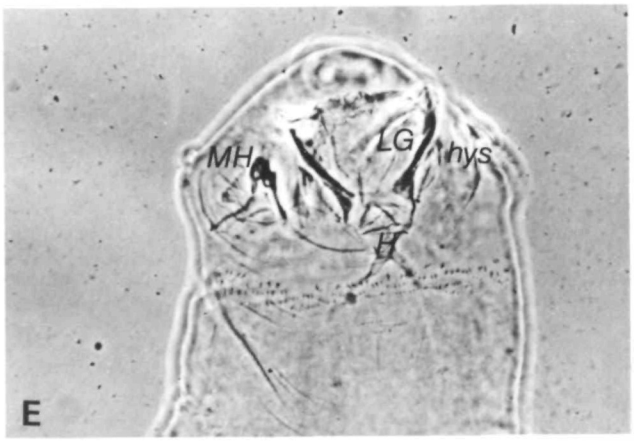
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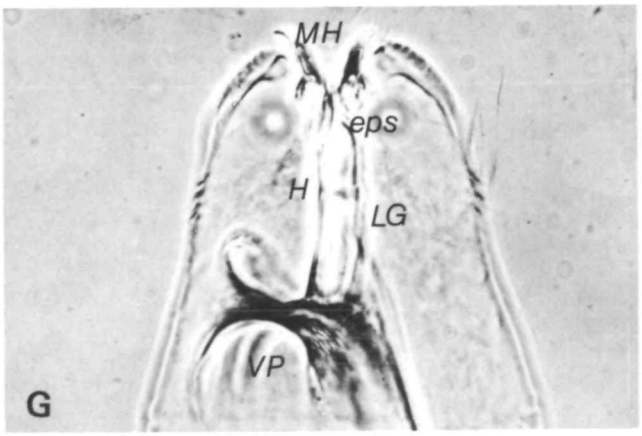
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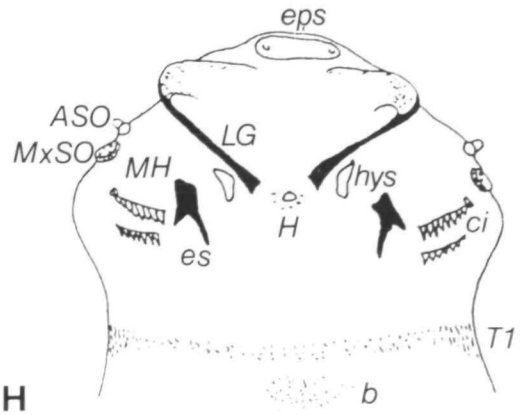
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Several other observations are worth noting. Particularly with heat shocks around 4 or 5 h of development, the maxillary sense organ could occasionally be induced to split into two groups of papillae, presumably corresponding to the dorsomedial and dorsolateral papillae (Keilin, 1915; Hertwick, 1931). This separation also occurs in some homeotic phenotypes, such as *Deformed* (Frederick & Denell, 1982; Regulski *et al.* 1987). Simultaneously, these papillae could also be separated from their associated antennal sense organ so that they came to lie on opposite surfaces of the head (Fig. 2A). This effect was seen in around 80 % of embryos treated during the susceptible period, although to different degrees on the left and right sides of individual embryos. The mouth hooks, while generally not morphologically normal, were clearly identifiable in the posterior ventral regions of all preparations and were always associated with a small rod of cuticle which we identified as the ectostomal sclerite. The cirri and ventral organ were also always tightly associated with these structures. We have not detected any differences in the number of papillae per cirrus after heat shocks at different stages of development. The hypostomal sclerites also appeared as curved rods, generally attached to the lateralgräten, although with heat shocks around 7 h they could be induced to lie adjacent to the latter structures. The H-piece was often difficult to detect, probably corresponding to a patch of diffuse pigmented material that lay between or at the join of the lateralgräten (Fig. 1E).

(B) *Effects on the dorsal surface of the head*

The cuticular structures most affected by induced ectopic expression of Antp were those deriving from the anteriormost (labral and acronal) portions of the head anlage (see Jürgens *et al.* 1986), that is, the CPS and dorsal sclerites, as can be seen in Fig. 2. In all preparations, a large, shield-like pigmented structure bearing two cuticular pores could be identified dorsally and anteriorly (Fig. 2D, arrowhead). Where head involution was more advanced, it was seen to follow the CPS into the atrium and thus almost certainly corresponds to the unpaired epistomal sclerite. Posterior to this structure, a range of other pigmented plaques could be observed, which varied with the time and extent (see section D below) of heat-shock induction of *Antp* expression. In embryos treated at the onset of gastrulation (3 h), a quite distinct ridge of strongly pigmented sclerite immediately anterior to the dorsal prothoracic belt of hairs could be seen, in addition to a more anterior broad patch (or patches) of sclerite. The identity of these structures is unknown, but they may be regarded, at least in part, as remnants of the unpaired labrum, or median tooth, which we were not able to identify unambiguously in any of the preparations from embryos treated in the first 7 h of embryogenesis.

In addition, two new belts of denticles could be induced by ectopic expression of *Antp*. The more posterior belt was only produced by treatments in the first 5 h of development, being best defined if the heat shock was administered at 3 h. It actually consisted of two patches of denticles, with characteristics of both ventral and dorsal abdominal denticle belts (Fig. 2A) on either side of the pigmented material. The more anterior belt, by contrast, seemed only to consist of ventral-type denticles and was most strongly induced by heat shocks around 6 h of development (Fig. 2B). In favourable preparations, it was possible to follow this belt around the anterior tip of the noninvoluted head. In some specimens, curiously, the two types of denticle belt were asymmetrically distributed, one of each forming only on either side of the embryo.

(C) *Disruptions of the prothoracic denticle belts*

The clearest example of a change in segmental identity observed in embryos after heat-shock induction of *Antp* was a transformation of prothoracic (T1) towards mesothoracic (T2) identity. In wild-type embryos, the prothoracic segment bears two belts of ventral denticles, an anterior belt four or five rows broad, which extends around the embryo linking up with the dorsal hairs, and a more posterior patch, or beard, of thick denticles. This situation also pertains to embryos heat shocked at 3 h of development (Fig. 3G), despite their failure of head involution. However, embryos treated between 5 and 8 h of development (i.e. during and after germ-band extension) showed a distinct reduction in the number of denticles in the prothoracic beard, as well as a less severe reduction in the density of the anteriormost band (Fig. 3H,J).

These reductions were quantified and the results are shown in Table 1. The prothoracic beard of denticles was scored as 'normal' if at least five rows of denticles were present, 'missing' if less than 20 denticles, and 'reduced' if there were an intermediate number of denticles. Representative camera-lucida drawings of these phenotypes are shown in Fig. 3G, J and H, respectively. Reduced prothoracic beards were observed in over 85 % of embryos after 20 min heat shocks at 7 h of development in all three heat-shock–*Antp* strains examined (but no reductions were observed after dechorionation, or in HTC-1 or K104 control embryos). In the H4 strain, 60 % of these embryos showed 'missing' prothoracic beards, indicating an almost complete transformation of pro- to meso- (or meta-) thorax in response to incorrect Antp protein production.

We have also observed a less severe reduction of some abdominal denticle belts in H4 (results not

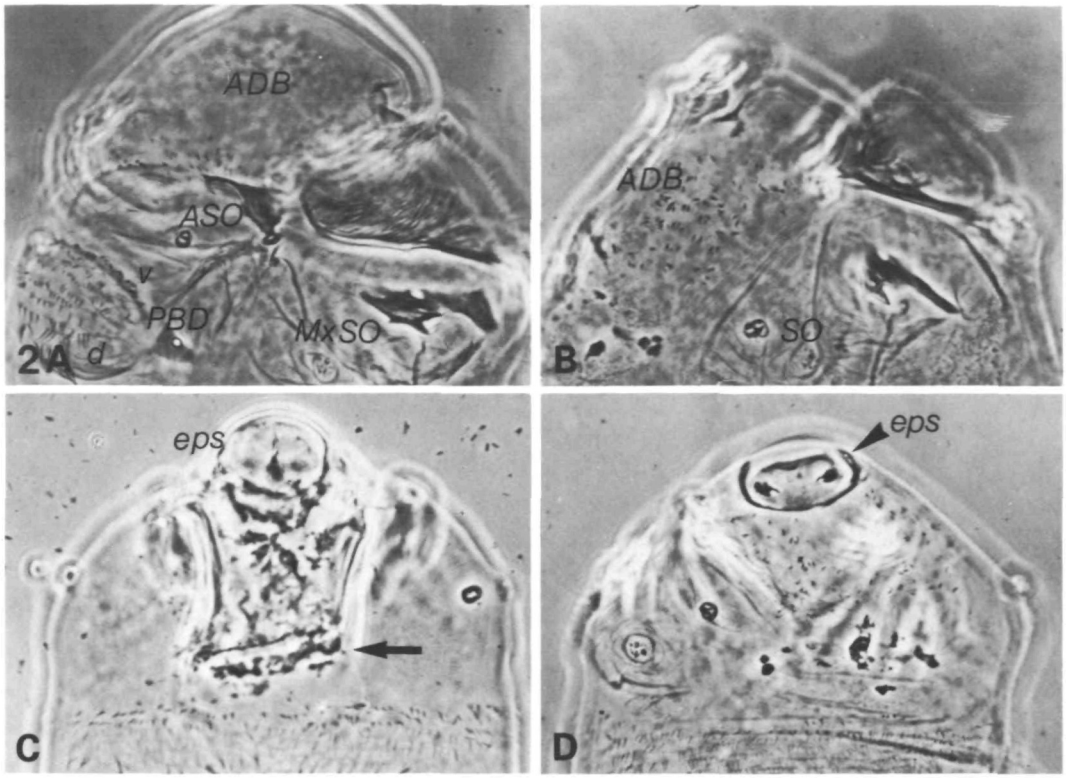

Fig. 2. Effects of heat-shock induction of *Antp* on the dorsal surface of the embryonic head. ×75. (A) Lateral view of the head of an H4 first instar larva (dorsal is on the left) which had been heat shocked for 20 min at 4 h of development, photographed using phase-contrast optics. Dorsal (*d*) and ventral (*v*) type denticles on the posterior denticle belt are indicated; a few denticles forming a small anterior denticle belt (*ADB*) are also visible. (B) Similar view of an H4 larva treated at 6 h of development, showing a well developed anterior denticle belt consisting solely of ventral type denticles. Note that the sensory organs (*MxSO* and *ASO*, abbreviated *SO*) are no longer separated as in A. (C) Dorsal view of an H4 larva heat shocked for 10 min at 7 h of development, showing a posterior ridge of cuticle (arrow), patches of pigmented material probably corresponding to the labrum, and the epistomal sclerite at the anterior tip of the embryo. (D) Dorsal view of an H4 larva heat shocked for 20 min at 7 h of development, showing more distinct pieces of pigmented cuticle, and an anterior belt of denticles. Abbreviations, Fig. 1.

Table 1. *Analysis of effects of heat shock induction of* Antp *on prothoracic denticle belts*

	Strain							
	H4 Treatment				H22 Treatment		H45 Treatment	
Effect	NT	DC	10′	20′	10′	20′	10′	20′
Normal (Fig. 3G)	94	103	15	2	48	1	40	16
Reduced (Fig. 3H)	0	0	75	47	52	73	34	71
Missing (Fig. 3J)	0	0	1	73	0	12	0	19
Total embryos	94	103	91	122	100	86	74	106
% not normal	0	0	84	98	52	99	46	85
% missing	0	0	1	60	0	14	0	19

NT, no treatment; DC, dechorionated; 10′, 10 min heat shock at 37°C; 20′, 20 min heat shock at 37°C.

shown). Heat shocks for 20 min at 7 h of development cause the complete deletion of the anteriormost row of denticles in the A2 denticle belt of 33% of H4 embryos. By contrast, similar treatments in other heat-shock–*Antp* strains and, in K104, HTC-1 and ry^{506} embryos, cause slight reductions in the number of denticles in this row in the majority of embryos. While the strong reduction is specific to excessive overproduction of Antp protein, the minor reductions are not, and consequently it is uncertain

whether this abdominal effect can be solely attributed to incorrect expression of *Antp*. No effects on development of the most posterior embryonic structures, the telson, have been observed.

(D) *Effects of different lengths of embryonic heat shocks in different strains*

In general, the severity of the effects on embryogenesis were proportional to the length of the heat shock applied and thus presumably to the amount of protein produced. The phase-contrast photographs and corresponding camera-lucida drawings (indicating the important cuticular structures) in Fig. 3A–F illustrate the typical effects of 5, 10 and 15 min heat shocks at 37°C after 7 h of development. Clearly head involution advanced considerably if only a brief heat shock was given, but was successively more inhibited with longer heat shocks, although heat shocks longer than 20 min had no further effects other than lethality. The effects produced by shorter treatments were more variable than with longer heat shocks, although 5 min was sufficient to prevent hatching in all H4 embryos. Similar results were obtained at all stages of embryogenesis. On the dorsal surface of the head, longer heat shocks tended to result in larger, more differentiated denticle 'belts', and smaller, more clearly defined patches of sclerite in place of the large smears of pigmented cuticle seen after short heat shocks (compare Fig. 2D with C). Both effects probably represent stronger transformations.

The strongest effects were seen in the strain H4. Three strains (H22, H45 and H36) produced similar degrees of transformation, which were equivalent to those obtained with heat shocks of half of the length in H4. Immunofluorescence staining of embryos (data not shown) and Western blotting (Fig. 8) shows that these differences can largely be explained by the amount of Antp protein produced in the different strains.

(E) *Late embryonic induction of* Antennapedia *causes larval lethality*

Since cuticle deposition in *Drosophila* occurs at approximately 12–15 h of development (Campos-Ortega & Hartenstein, 1985), and it has previously been argued that external segmental identity might reflect the state of activity of the various homeotic selector genes at that time (Struhl, 1983), we also examined cuticular phenotypes of larvae heat shocked in the second half of embryogenesis. We could not detect any effects on head involution or denticle belt formation with 15 min heat shocks throughout late embryonic development. Nevertheless, up until the last 2 h of embryogenesis, ubiquitous induction of Antennapedia protein had a marked effect on mortality. For treatments at 13, 15, 17 or 19 h of development, all embryos were able to hatch, but only about 20% of them survived to pupariate and of these only half emerged as apparently normal adults. (In addition, surprisingly, heat shocks at 17 h of development led to larval lethality in the HTC-1 control strain, without causing any visible effects on the cuticle.)

(F) *Degradation and amount of the induced Antennapedia protein in embryos*

Since we observed specific cuticular defects associated with heat shocks at each stage of embryogenesis, we wanted to determine how long the Antp protein persisted after induction. Immunofluorescence staining of embryos revealed that Antp protein reaches its highest levels after a 1 h recovery from a 20 min heat shock (Fig. 4A,B). The staining is nuclear and of slightly stronger intensity than that in the T2 nuclei when Antp protein first appears at the germ-band extension stage (Wirz *et al.* 1986; compare Fig. 4E,F). After 2 h recovery, there is a low level of background staining, particularly in the presumptive thoracic and abdominal regions (Fig. 4D), but no nuclear staining is detectable. 3 h after a 20 min heat shock, no staining above background can be detected. Attempts to visualize Antp protein on Western blots using embryos heat shocked in the same way were unsuccessful. Taken together with the fact that 2 min heat shocks in H4 were sufficient to induce some developmental defects, this data suggests that the anterior segments of embryos are particularly sensitive to Antp protein, which need only be present in levels equivalent to those normally present in T2 for it to cause severe developmental defects.

(G) *Expression of Scr protein after heat shock*

The transformation of prothorax towards posterior thorax described above is similar to that observed in embryos deficient for the *Scr* gene (Wakimoto & Kaufman, 1981). We therefore sought to determine whether the ectopic expression of *Antp* in the prothorax had any effect on Scr protein production, by immunodetection of Scr protein after heat shock at the germ-band-extension stage of H4 embryos. Anti-Scr antibody was detected using a peroxidase-coupled secondary antibody. By the end of germ-band retraction, Scr protein in wild-type embryos can be detected in the anterior portion of the prothorax, in the labial segment and a few posterior cells of the maxillary segment (Fig. 5A; see also Riley *et al.* 1987). Representative H4 embryos allowed to recover for 1, 2 and 3 h after a 20 min heat shock at 7 h of development are shown in Fig. 5B,D,F, respectively, by comparison with identically treated K104 embryos (Fig. 5A,C,E). It is possible that staining in the prothorax after 2 h recovery is, in some embryos,

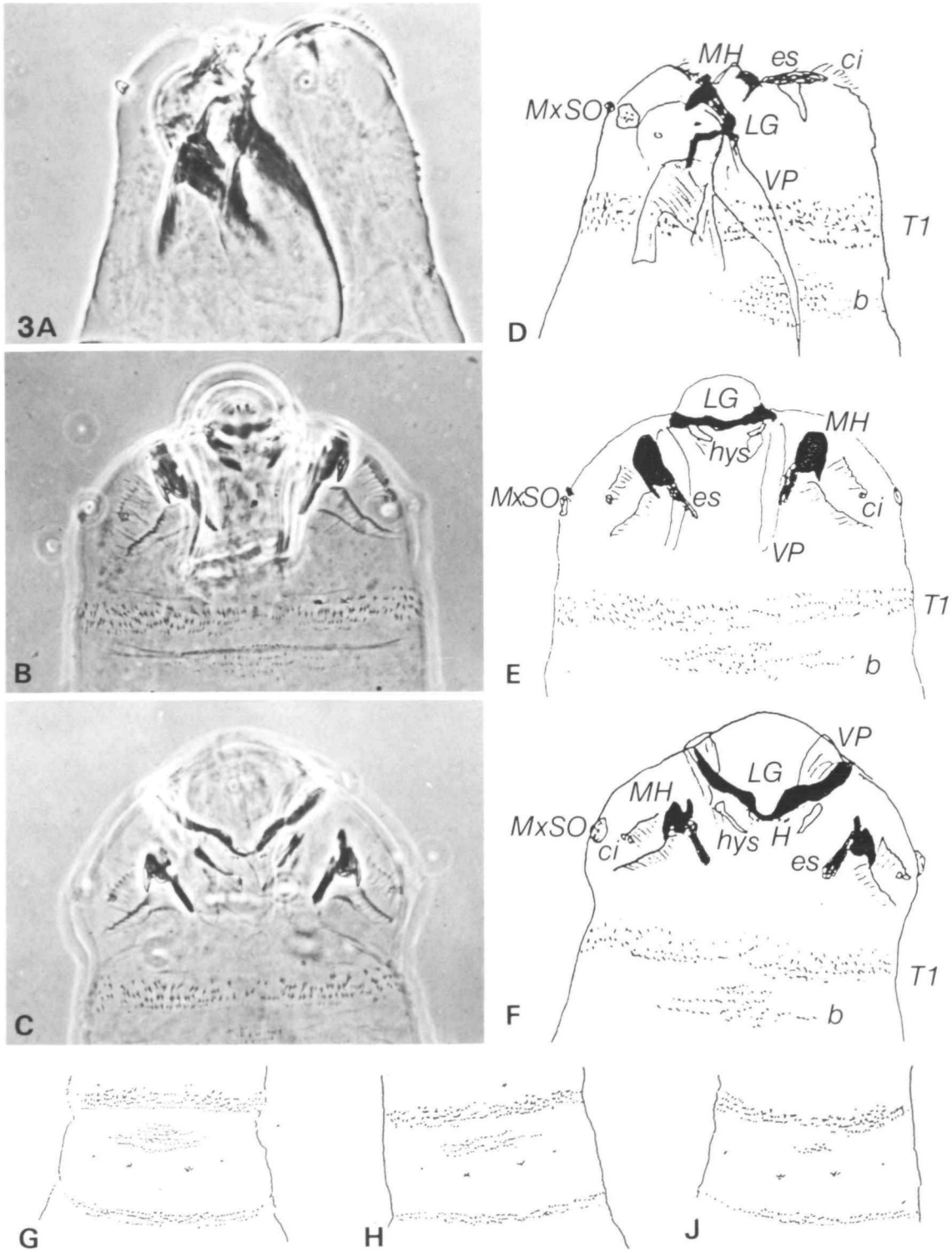

Fig. 3. Effects of different lengths of heat-shock induction of *Antp* on head and prothoracic development. ×75. (A) Ventral view of the head of an H4 first instar larva that had been given a 5 min heat shock at 7 h of development, photographed using phase-contrast optics. B and C similarly show heads of larvae heat shocked at the same time, for 10 min and 15 min, respectively. D–F are corresponding camera-lucida drawings. Note that head involution is even more inhibited after a 20 min heat shock at 7 h of development: compare the positions of the LG in Fig. 1A with C. (G,H,J) Camera-lucida drawings of the ventral prothorax of representative embryos showing 'normal', 'reduced' and 'missing' phenotypes of the beard of denticles, respectively (see text for explanation). The bilaterally paired organs posterior to (below) each beard are the Keilin's organs and ventral pits. Abbreviations, Fig. 1.

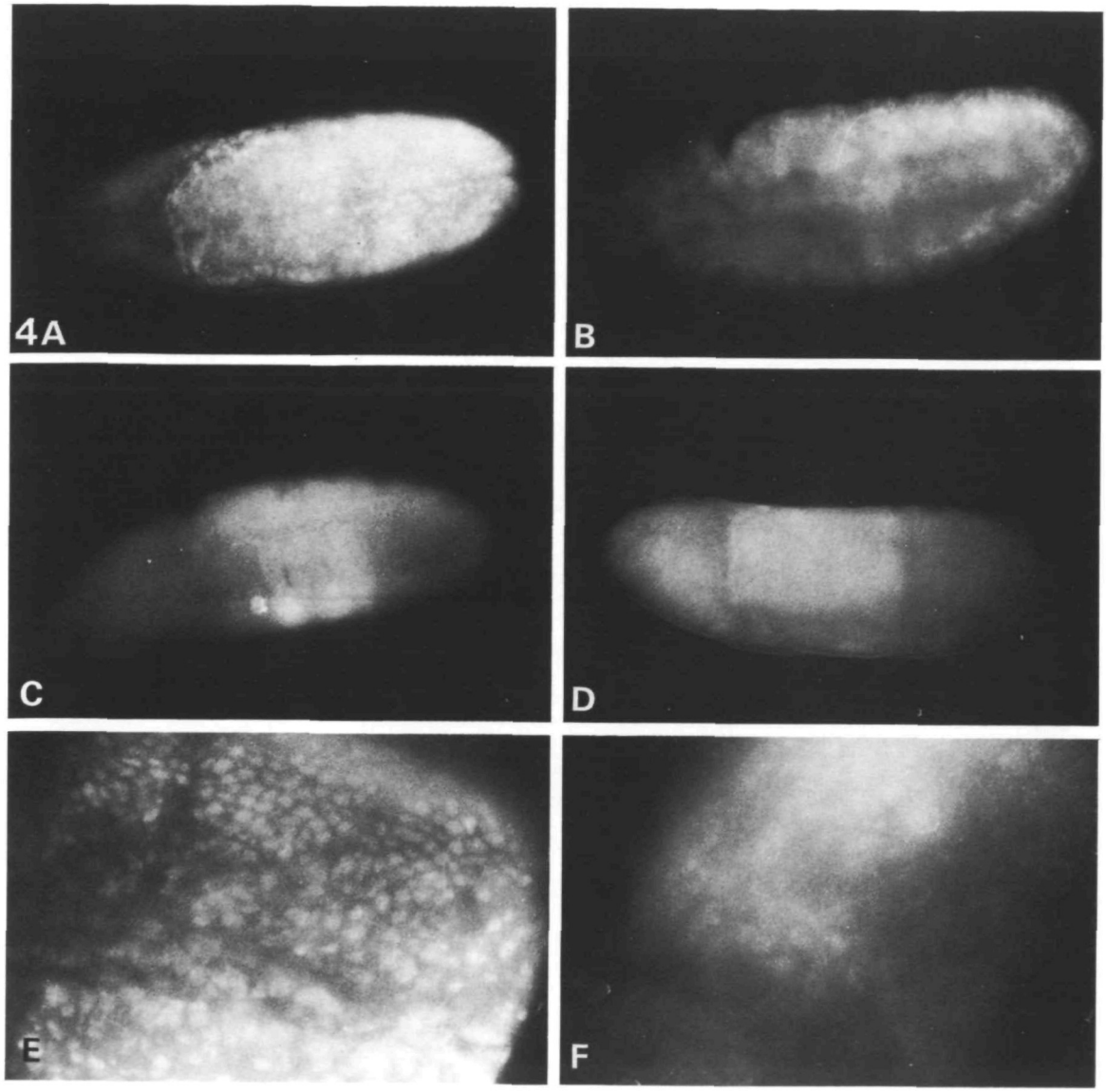

Fig. 4. Degradation of Antp protein after heat-shock induction in embryos. ×50. (A,B) Germ-band-extension stage dorsal and lateral views respectively of H4 embryos 1 h after a 20 min heat shock at 5 h of development. Nuclear staining is detectable throughout the embryos and is particularly strong in the germ-band regions. (C) Germ-band extension H4 embryo without heat shock (lateral view), showing initial Antp protein appearance in T2 and T3. (D) Germ-band-extension stage H4 embryo (lateral view) 2 h after a 20 min heat shock, showing light staining slightly above background throughout the embryo. (E,F) High-magnification views of the embryos shown in A and C, illustrating that nuclear Antp staining 1 h after a 20 min heat shock is slightly stronger than its intensity when it normally first appears in T2. ×100.

slightly reduced in H4 embryos by comparison with K104 control embryos (compare Fig. 5D with C), as might be predicted if Antp can suppress *Scr* expression (Riley *et al.* 1987). However, after heat-shock induction of *Antp*, in all embryos examined and after all stages of recovery, there are still significant levels of Scr protein detectable in the regions where it is normally expressed. Since Antp protein is only detectable for 2 h after a 20 min heat shock, we conclude that both Scr and Antp protein are present simultaneously in the prothorax during the time when the prothoracic transformation is determined.

(H) *Effects on leg development*

It has previously been reported that extensive (2 h) heat shocks in H4 flies during the third instar larval stage cause a strong transformation of antennae towards mesothoracic legs (Schneuwly *et al.* 1987*a*). To define this response better and to determine whether the development of any other imaginal discs could be altered by transient ectopic expression of the

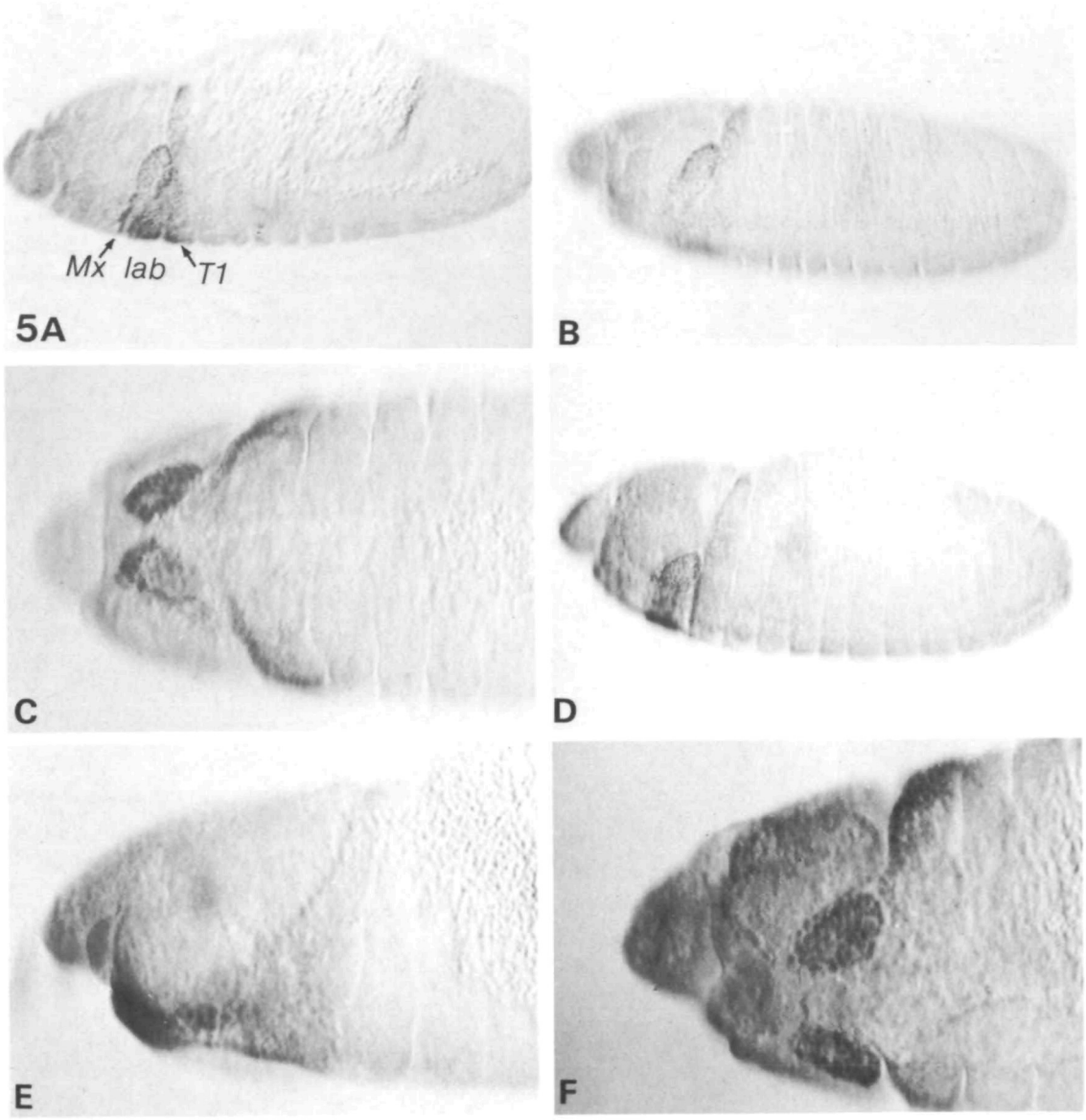

Fig. 5. Production of Scr protein after heat-shock induction of *Antp*. ×50. (A) Expression of Scr in K104 control embryos at the germ band retraction stage (8½ h), 1 h after a 20 min heat shock, is confined to cells of the anterior prothoracic (*T1*), labial (*lab*) and posterior maxillary segments (*Mx*). (B) An identical pattern of Scr expression is seen in H4 embryos treated in the same way, that is, showing Scr staining when Antp is expressed at a maximum. (C) Lateral view of K104 embryo 2 h after a 20 min heat shock at 7 h of development. (D) Ventral view of H4 embryo treated as in C. (E) Lateral view of K104 embryo 3 h after heat shock and (F) ventral view of an H4 embryo at the same stage. Staining in the prothorax in D is possibly slightly less than in C, although this is variable from embryo to embryo. In F, the labial cells that stain have remained lateral and posterior, by comparison with those in C and E, indicating that the cell movements required for head involution have not occurred.

Antp protein, we heat-shocked developing insects for 30, 45 or 60 min at 4 h intervals throughout the three larval and the pupal stages. It was found that 45 min heat shocks are sufficient to produce most of the transformations observed, although to a lesser degree than longer heat shocks, which also cause larval or pupal lethality when applied at a number of stages. The results are summarized in Table 2. No transformations or alterations are seen in HTC-1 or K104 control flies under the same conditions.

Larvae heat shocked in the first 12 h after hatching show little effect on development. However, in late first instar larvae, a strong induction of *Antennapedia* leads to late larval or pupal lethality. By delivering a

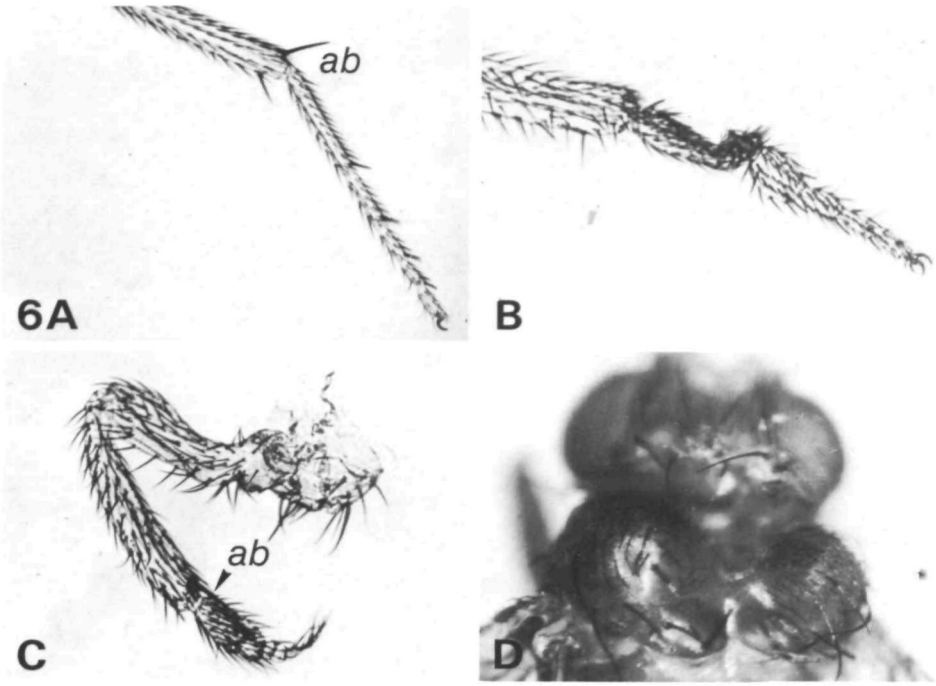

Fig. 6. Adult thoracic defects induced by heat-shock overexpression of *Antp*. (A) Wild-type mesothoracic leg. ×25. (B) Kinked metathoracic leg induced by 1 h heat shock in H4 mid-third instar larva. (C) Almost complete transformation of antenna to mesothoracic leg (shown by presence of apical bristle, *ab*) induced by multiple 1 h heat shocks in H4 third instar larva. ×30. (D) Cleft thorax phenotype induced by 1 h heat shock in H45 late third instar larva.

Table 2. *Summary of effects of heat shock induction of* Antp *during H4 development*

Embryonic stage	Effect of 20 minute heat shock
3 h	failure of head involution; posterior dorsal cephalic band of denticles
5 h	failure of head involution; anterior dorsal cephalic band of denticles
7 h	failure of head involution; prothorax-to-mesothorax transformation
9 h	slight failure of head involution
11–13 h	no cuticular defects, but embryos fail to hatch
15–19 h	50 % hatch, but can't move or eat
21 h	develop to normal adults
larval stage	**Effect of one hour heat shock**
1st instar	develop to normal adults
early 2nd instar	occasional minor leg defects
mid 2nd instar	larval/pupal lethal
late 2nd instar	arista to claw and tarsus transformation
0–4 h 3rd instar	third antennal segment to tibia (with apical bristle) transformation
4–8 h 3rd instar	second antennal segment to femur transformation
early to mid 3rd instar	cephalothorax transformation, occasional transformation of maxillary palpus and vibrissae
mid 3rd instar	no effect generally, some leg defects
mid to late 3rd instar	H45: cleft thorax H4: lethal
late 3rd instar	lethal
pupae	lethal

less-severe heat shock, it was found that some alterations of hind leg development can be induced, as is shown in Fig. 6B. Abnormal metathoracic (hind) legs arise in less than 20 % of the adult flies examined and, characteristically, involve kinks in parts of the different leg segments, especially the distal segments, rather than transformations of segmental identity: the normal bristle patterns of legs from the segment of interest were always observed. Heat treatments in early- to mid-third instar larvae can also cause leg abnormalities. These effects were seen in all legs on all three thoracic segments. In addition, we have observed very strong cleft-thorax defects after a 1 h heat shock in H45 mid- to late-third instar larvae (Fig. 6D). In some animals, the dorsal mesothorax is almost split into two pieces, which may partially account for the lethality of the same treatment in H4 larvae.

(I) *Distal-to-proximal transformations of antenna towards leg*

The wild-type antenna of *Drosophila*, shown in Fig. 7A, consists of three well-developed segments, the most distal of which (the third) bears the arista which is itself mounted on a basal cylinder. Careful timing of heat shocks relative to the third larval moult reveals that the first stage at which the developing antenna is susceptible to the activity of Antp is in the last 4 h of the second instar larva. Heat shocks applied at this time typically cause a transformation of the most distal portions of the antenna towards distal leg structures (Fig. 7B). Stronger heat shocks lead to an

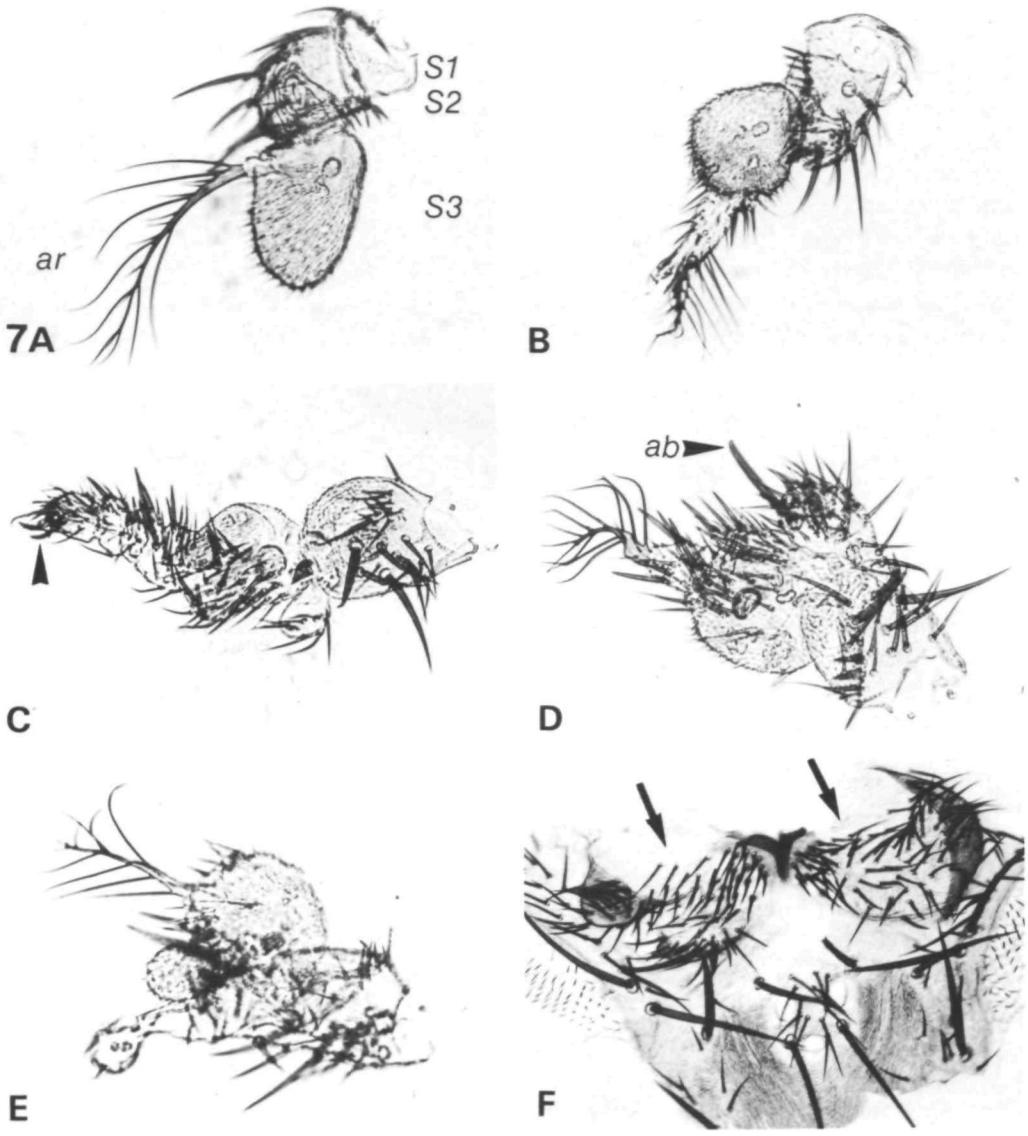

Fig. 7. Distal-to-proximal transformation of antenna to leg induced by successive heat-shock induction of *Antp* in early third instar larvae. (A) Wild-type antenna, showing the three conspicuous antennal segments (*S1–S3*), and the arista (*ar*): proximal portions are to the top. (B) Weak arista to metatarsus transformation, leaving the remainder of the antenna normal, induced by 1 h heat shock in late second instar H4 larva. (C) Strong arista to tarsus transformation induced by same treatment as in B, showing development of a claw and distinct metatarsal segments. (D) 3rd antennal segment to tibia and mesothoracic tarsus transformation, including development of an apical bristle *ab*, induced by 1 h heat shock in early third instar H4 larva. (E) 2nd antennal segment to femur transformation, leaving arista and 3rd antennal segment virtually normal, induced by 1 h heat shock in 4–8 h third instar larva. (F) Strong cephalothorax transformation induced by 1 h heat shock in 4–8 h third instar larva. The bristles indicated by the arrows, and the bulges they grow on, are not seen in wild-type heads.

almost complete transformation of the arista to a claw (Fig. 7C, arrowhead); in addition, the basal cylinder of the arista often enlarges and becomes segmented to resemble metatarsal segments (Fig. 7C). Rarely, however, are alterations to the remainder of the antenna observed.

Thereafter, heat shocks at successively later stages of development lead to transformations of successively more proximal portions of the antenna. Larvae heat shocked just after emerging from the third larval moult show a less-severe reduction of the arista, as well as modifications of the 3rd antennal segment, such as elongation and bristle induction. Slightly later in development still, severe alterations of this segment are induced, generally resulting in the appearance of three or four bulges of tissue as if the segment was elongating in more than one direction. In addition, the antennae become covered with bristles,

particularly in the valleys between the bulges; often, one bulge also bears a large terminal bristle which presumably corresponds to the apical bristle characteristic of mesothoracic legs (Fig. 7D, arrowhead). Heat shocks applied to 12 h third instar larvae sometimes induce growths on the second antennal segment, leaving the third segment and arista virtually intact (Fig. 7E). Later still in development, transformations are far more mild, resulting in only slightly reduced aristae and the appearance of just a few bristles on the 3rd antennal segment.

Apart from the thoracic defects described above (Section H), the only other tissues affected by ectopic expression of Antp also derive from the eye–antennal disc. Occipital transformations can be induced by transformations in early- to mid-third instar larvae, typically resulting in a large patch of bristles on the posterior portion of the head (Fig. 7F), which closely resembles the cephalothorax transformation observed in the $Antp^{Clx}$ allele (Duncan & Lewis, 1982). Occasionally, the maxillary palps also fail to develop normally, although often only on one side of the head. Multiple heat shocks administered to third instar larvae can result in an almost complete transformation of antenna towards leg (Fig. 6C), and dorsal head towards thorax. Heat shocks of H4 late in larvae and in pupae are usually lethal. We have not yet detected any alterations in abdominal development, but cannot rule out the possibility of minor alterations in bristle patterns which might indicate a change in segmental identity.

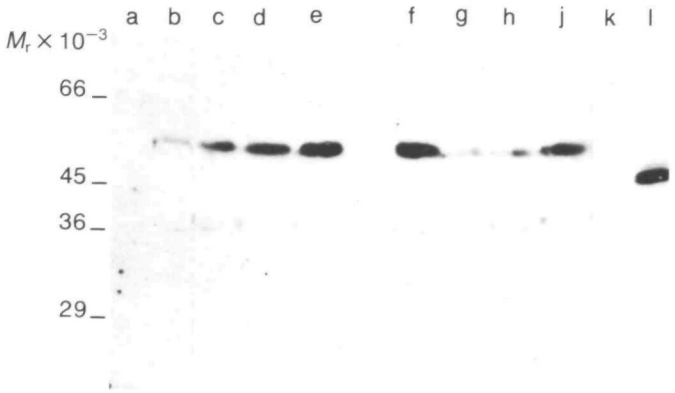

Fig. 8. Western blot of Antp protein production after different lengths of recovery from heat shock and in different transgenic strains. Antp protein from the equivalent of one late third instar larvae, after SDS–PAGE and transfer to a nitrocellulose filter, was detected using a rabbit anti-Antp antibody coupled to ^{125}I-labelled protein A, as described in Materials and methods. The autoradiogram was obtained by 2-day exposure to preflashed X-Omat film. Similar results were obtained in four independent experiments. (a) H4, non-heat shock. For the remaining lanes, larvae were heat shocked for 1 h and allowed to recover for the indicated times. (b) H4, 8 h. (c) H4, 4 h. (d) H4, 2 h. (e,f) H4, 1 h. (g) H22, 1 h. (h) H36/CyO (hemizygous), 1 h. (j) H45, 1 h. (k) ry^{506}, 1 h. (l) K104, 1 h. Molecular weight markers, M_r, indicate that the protein(s) migrate with higher apparent molecular weights than would be predicted from the *Antp* sequence (55×10^3 compared with 42×10^3 predicted for full-length protein; B. Dalle Carbonare and W.G., unpublished data).

(J) *Antennapedia protein production in third instar larvae*

Strong transformations of antenna to leg were only observed in the H4 strain, 1 h heat shocks in other transgenic heat-shock–*Antp* strains merely causing the induction of extra bristles on the antenna. As is shown in Fig. 8 (lanes F,G,H and J), a Western blot of Antp protein from different strains of third instar larvae after 1 h recovery from a 1 h heat shock, this result is most readily explained by the fact that H4 produces more protein than the other strains. The protein produced by the K104 strain is smaller than that in the heat-shock–*Antp* strains, indicating that it is truncated as expected (lane L). Interestingly, the H45 strain (lane J) produces significantly more protein than H22 and H36 (lanes G and H, respectively; the same result has been obtained in four independent experiments), but does not produce stronger phenotypic changes. It is unlikely that this is because the heat-shock promoter in H45 is inactive in larval heads, since immunofluorescence staining of embryos also suggested that it produces more protein than the other two strains, without having a concomitant phenotypic effect. Perhaps H4 has stronger effects because it may be a low-level constitutive producer of Antp protein. The different apparent strengths of the heat-shock promoters in the different strains probably reflect position effects of the sites of chromosomal insertion.

Fig. 8 (lanes A–E) also show that the Antp protein (and mRNA) degrades to low levels 8 h after a 1 h heat shock. Comparison of lane B with lane G (H22) suggests that after 8 h of recovery, the protein is no longer present at levels sufficient to cause developmental transformations in the antennal imaginal disc. Since it is not possible to detect Antp protein production in wild-type eye–antennal imaginal discs, we cannot say how many fold the increase in Antp protein production required to cause transformations is. However, from longer exposures of the filter in Fig. 8, and other data (B. Dalle-Carbonare and W.J.G., unpublished) we estimate that at least a tenfold increase in Antp production in total third instar larvae is required to induce the transformations described here. This is in sharp contrast with the situation in embryos, where a small amount of Antp

protein appears to be sufficient to cause drastic developmental defects.

Discussion

In order to define more precisely the effects of ectopic expression of a homeotic gene, we have examined the phenotypic effects of inducing Antp protein to varying extents at defined stages throughout embryonic, larval and pupal development. When overexpressed during the first 10 h of development, and in all cells of the embryo, Antp causes a failure of head involution, reductions in prothoracic denticle belts and the appearance of a number of new cuticular structures in the head. In later embryonic development, and with extensive inductions throughout larval and pupal development, the protein caused lethality often without having any visible effects on the adult cuticle. Inductions in second and third instar larvae led to malformation of some thoracic and antennal structures, causing a distal-to-proximal transformation of antenna towards mesothoracic leg.

One criticism of the general method described here is that heat shock alters the total pattern of gene expression in the developing organism for a short period (see Chomyn *et al.* 1979) and that the system is therefore in a sense artificial. Mitchell & Lipps (1978), for example, have shown that phenocopies of various bristle mutations can be induced by 41 °C heat shocks at very precise stages of development. Furthermore, Peterson & Mitchell (1987) have more recently argued that genes in a heterozygous state are particularly susceptible to the effects of repression of transcription which occurs after heat shock. However, we examined the effects of heat shocks on a number of control strains, including the initial ry^{506} stock used for transformation, a transformant containing the heat-shock vector alone, and one expressing a truncated *Antp* gene; but have not detected any of the effects described in this report in any of these strains. While it can still be argued that the general cellular environment is altered by heat shock, our conditions seem to be mild enough not to visibly disrupt development.

Abnormally expressed Antennapedia *influences the determination of segmental identity – the competition hypothesis*

Precise control of the timing and duration of heat shocks in developing heat-shock–*Antp* transformed flies has allowed us to characterize the effects of ectopic Antennapedia production in terms of segmental transformations. Jürgens *et al.* (1986) have used u.v. laser microirradiation to examine the segmental organization of the head of the *Drosophila* embryo. We have found that the structures, to which they assigned common segmental origins, are affected to similar extents by overexpression of *Antennapedia*. There would appear to be a general anterior-to-posterior gradient of severity of effect of ectopic Antennapedia, the protein causing clear reductions of anterior structures (especially the cephalopharyngeal skeleton), while merely preventing the involution of more posterior structures such as the maxillary organs.

The experiments reported here do not directly address the mechanism of action of *Antp* in the determination of segmental identity, but they do provide further support for the hypothesis that such identity is dependent upon the relative activities of the various homeotic selector genes in each segment. In particular, it seems likely that the selector genes act combinatorially (Struhl, 1982), probably through competition with one another, to determine segmental identity. But why are anterior (head) structures apparently more susceptible to overexpressed *Antp* than posterior (abdominal) structures?

Trivially, this observation may merely be an artifact of the fact that abdominal segments are more similar to thoracic segments (where *Antp* is normally required during development) than are head segments: many of the functions performed by ectopically expressed Antp might already be performed by the BX-C genes in the abdomen.

Alternatively, competition amongst the selector genes may not be equal – the abdominal selector genes might be considered to be incompletely epistatic to *Antp*, which is in turn incompletely epistatic to head selector genes. Part of the reason why *Ubx*, for example, seems to be epistatic to *Antp* certainly relates to the existence of crossregulatory interactions amongst the homeotic selector genes. Thus, Hafen *et al.* (1984) have shown that *Ubx* is able to repress the transcription of *Antp* posterior to parasegment 5. Similarly, Riley *et al.* (1987) have recently demonstrated that at least in some cells, *Antp* alone is capable of repressing *Scr*.

Riley *et al.* (1987) also examined two classes of embryos genetically null for the *Scr* gene, which show strong pro- to mesothoracic transformations and were unable to detect any Scr protein by immunofluorescence staining. However, we have shown here that a similar transformation can also be caused by the overexpression of Antp protein in the prothorax, without abolishing the expression of Scr protein in the same prothoracic cells. Within the limits of resolution, given the considerable variation in staining intensity in different embryos, it remains possible that there is a slight reduction in Scr expression in the prothorax 2 h after heat-shock induction of *Antp*. The failure to observe repression of *Scr* in our experiments may reflect a requirement for continuous

production of high levels of Antp protein, or could imply that other factors apart from Antp itself are required for it to repress *Scr* in regions where *Scr* is normally expressed. In any case, during the period when Antp protein is detectable in the prothorax after heat-shock induction, Scr protein also remains detectable at approximately wild-type levels. This suggests that the homeotic proteins can compete with one another on a level independent from crossregulation, probably in the direct control of the transcription of downstream genes.

An explanation for the semiepistatic effects described above could relate to the fact that all of the selector genes identified so far contain a conserved nucleotide sequence, the homeobox, which is thought to encode a DNA-binding domain (Shepherd *et al.* 1984; Laughon & Scott, 1984; Desplan *et al.* 1985; Gehring, 1987). The relative dominance of each selector gene might in part depend upon its affinity for the promoters of realisator genes and of other homeotic genes. Alternatively, portions of the homeotic proteins outside of the DNA-binding domain may be crucial in determining the ways in which these proteins control gene expression, possibly in their regulation of protein–protein interactions. An understanding of why ectopically expressed Antp protein is apparently incapable of competing effectively with bithorax-complex proteins awaits a molecular analysis of the roles of the different domains of the homeodomain-containing proteins and biochemical studies of their DNA-binding properties.

It is also clear that transiently induced Antp is not by itself capable of transforming homologous structures, such as first and third legs, in the presence of selector genes normally expressed in those discs. While ectopic induction of Antp can cause the antennae to transform towards mesothoracic legs (as evidenced by the appearance of apical bristles: Schneuwly *et al.* 1987*a*, and this report), in no cases have we observed replacement of pro- or metathoracic leg parts, such as the sex combs and transverse rows, by mesothoracic parts. This would argue either that such transformations require prolonged Antp activity (unlikely in view of the antennal leg identity), or that *Scr* and *Ubx* are epistatic to *Antp* in the imaginal discs. Alternatively, as has been argued by Lewis (1978), *Antp* may just specify the ground state, which is postulated to be mesothoracic identity, and the expression of other selector genes modifies this ground state despite the activity of Antp protein. Nevertheless, at least in imaginal disc development, the stoichiometric ratios of selector genes are probably not the sole factors determining how they compete with one another to control downstream gene expression and so determine segmental identities.

Since different larval cuticular structures are affected by heat shocks delivered at slightly different stages of development, and we have shown by immunofluorescence staining that overexpressed protein rapidly degrades to undetectable levels, it is likely that the ectopic protein exerts its effects within 2 h of induction. Much of the effect of incorrect expression of *Antp* is no doubt to reprogramme developmental pathways, but it also seems to have direct effects on morphogenetic movements. We have found consistently that the failure of head involution which becomes evident in mature embryos is reflected in incorrect formation of the gnathal lobes between 6 and 12 h of development. This can be seen by comparing Fig. 5F with C and E. In control embryos at 10 h of development, the labial cells, which stain heavily for Scr protein, start to move ventrally and to involute into the thorax, but in H4 embryos, the same cells remain in a lateral position and fail to involute.

The effects on head development of incorrect expression of *Antp* can be most readily explained by postulating that the gene represses the activities of the head selector genes which are required for normal development of the head. Some of the defects described above are also observed in homeotic mutants such as *Deformed* (Regulski *et al.* 1986; Merrill *et al.* 1987) and *zerknüllt* (Wakimoto *et al.* 1984). Although we have not seen any direct replacement of cuticular structures which might be expected in homeotic phenotypes, this could be explained if any head-gene repression is only partial and/or temporary.

Since the major embryonic effect of ectopic *Antp* expression is to prevent head involution, it can be concluded that any genes it affects are not merely involved in determining cellular identities, but also in the control of morphogenetic movements. We cannot determine whether *Antp* directly disrupts one or other, or both, of these processes. The observed reductions in the cephalopharyngeal skeleton might be interpreted as repression of cuticle secretion and thus an example of altered cellular differentiation; but they could also result from abnormal cellular migration. Moreover, these two processes may not be separable: altered cellular migration will both be consequent upon and result in abnormal cell–cell contacts which may themselves influence cellular differentiation. It is difficult to explain the formation of extra ventral-type denticles, and their appearance on the dorsal surface of the head is equally puzzling: this could theoretically reflect repression of the dorsalizing gene *zerknüllt* (which is active in the head at the germ-band-extension stage: Doyle *et al.* 1986), and/or abnormal morphogenetic movements which bring ventral tissue to the dorsal surface of the head.

These issues might in part be resolved by the use of scanning electron microscopy and immunodetection of head gene expression once the relevant genes have been identified and cloned.

Antennal development occurs in a distal-to-proximal direction

Our initial experiments on unstaged larvae indicated that transformations could occur to different parts of the antenna. To determine whether there was any consistency in which structures transformed when, we induced *Antennapedia* at 4 h intervals throughout larval development. This demonstrated clearly that different portions of the eye–antennal imaginal disc become determined at different times. Previously, fate mapping of various imaginal discs has shown that the most distal structures of adult appendages generally map to the most-central regions of imaginal discs, while proximal structures arise from peripheral tissue (see generally Bryant, 1978; Schubiger, 1968, for the leg discs; Gehring, 1966, and Haynie & Bryant, 1986 for the antennal disc). Since in the experiments reported here the most distal antennal structure, the arista, was transformed by the earliest *Antp* inductions, and successively more peripheral structures (culminating in the occiput and maxillary palps) were transformed with later heat shocks, determination in the antennal disc appears to occur in a distal-to-proximal direction, which would correspond to a central-to-peripheral wave in the developing disc.

Ginter & Kuzin (1970) have similarly found that the arista is the first part of the disc to be ready to differentiate, although contrasting results were obtained in similar experiments by Gateff & Schneiderman (1975; see also Bryant, 1978). They transplanted discs from embryos of varying ages into ready-to-pupate larvae and found that there was a general tendency for more proximal structures to develop from transplanted discs taken from younger donors, which led them to conclude that the different portions of the antennal disc acquire competence to differentiate in a proximal-to-distal sequence. The reasons for the contrasting results is not clear, but the fact that aristae were observed in only 29 % of transplanted mature discs (Gateff & Schneiderman, 1975) suggests that there are artefactual constraints on that procedure. We have repeated our observations in five independent experiments, on three different strains of transformants, and the data appears to provide unambiguous evidence that determination in fact proceeds in a distal-to-proximal direction, with the determination of distinct parts occurring within a relatively short temporal window with a maximum length of approximately 8 h.

The results also shed some light on the mechanism of action of the Antennapedia protein. Since overexpression of the protein only affects certain structures at each stage, it would appear that there is no requirement for tight temporal control of its expression in the developing imaginal disc. For example, in the cells that give rise to the arista, the protein drastically influences development only when expressed in very late second instar larvae: ectopic expression later in development has little effect on distal antennal differentiation. While this situation represents the abnormal case of transformation of segmental identity, a similar argument might be applied to normal leg development, namely that the Antp protein will only be required in each segment of the developing imaginal disc during a 4–8 h temporal window.

This result also suggests an explanation for some of the differences in phenotypes of the different dominant *Antp* alleles: the protein might be overexpressed in the eye–antennal imaginal discs (see Jorgensen & Garber, 1987), at sufficient levels, at slightly different stages of development. For example, the $Antp^{Ctx}$ transformation might arise if the protein only attains a high enough level in mid-third instar larvae. In addition, the failure to detect tarsal transformations by genetic studies could be either because the protein is not ectopically induced early enough in any mutants, or because flies carrying such a transformation are generally unable to hatch (personal observations) and hence would be lethal even in a heterozygous state.

Conclusions

We have emphasized here the finding that antennal transformation occurs in a distal-to-proximal temporal direction, but it is equally important to note that persistent high level overexpression of Antp protein is required to yield strong transformations. Not only are most of the imaginal discs completely buffered against the effects of ectopic expression of a homeotic selector gene, but the susceptible antennal disc also shows considerable developmental inertia. Single heat shocks only ever affect discrete portions of the developing disc, implying that the disturbance to the developmental programme in the disc is short lived and the whole system recovers relatively rapidly.

This is perhaps in contrast with the situation in embryos where even a small heat shock can have drastic effects on development. The difference might be explained using the concept of generative entrenchment (Wimsatt, 1986; Rasmussen, 1987) which suggests that early developmental processes will be more constrained than those acting later in

development. Thus, alteration of the embryonic functions of Antp protein will seriously disrupt ontogeny, while alteration of Antp expression in the imaginal discs may not be so detrimental to the organism. Indeed, we have presented data that suggest that the precise spatial and temporal expression of *Antp* in imaginal discs does not need to be strictly controlled. These considerations suggest that small alterations in the domains of action of homeotic selector genes can be readily tolerated and that they may play a role in increasing evolutionary flexibility.

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